REMARKS

In the Official Action dated February 23, 2009, the Examiner has made the restriction final. Accordingly, Claims 1-12 are pending and under examination and claims 13-27 have now been withdrawn. Applicants reserve the right to file one or more divisional applications directed to the withdrawn subject matter.

Claims 4-6 have been objected to as allegedly in improper form. In response, Claims 4-6 have been amended to reflect proper claim drafting practice under 37 C.F.R. §1.75(c). New Claims 28 and 29 have been added and recite the limitations of the canceled portions of original Claims 5-6, wherein support therefore is explicitly found. No new matter has been added and no additional search is required.

Claims 1-12 have been rejected as allegedly lacking an enabling disclosure under 35 U.S.C. §112, first paragraph. Claims 1-3 and 7-9 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by Maliszewski (Pathol. Biol. 2001: 49:481-483 (hereinafter "Maliszewski").

This response addresses all objections and rejections of record. The pending claims have been clarified to reflect the recognition achieved by the present inventors that selectively elevating certain types of dendritic cells, particularly non-activated immature dendritic cells, using Flt-3 Ligand facilitates maintenance of a tolerogenic state in a subject. Accordingly, the present application is in condition for allowance.

Claims 1-3 and 7-9 have been rejected under 35 U.S.C. §102(b) as allegedly anticipated by Maliszewski. Maliszewski discloses immunotherapy based on expanding dendritic cells and/or activating dendritic cells in order to increase the immune response to an antigen, i.e. using Flt3L as an adjuvant, see p482, 2nd and 3rd paragraphs. The animal models

discussed are mouse tumor models, i.e. animals which already have a disease. The animals are treated with Flt3L and challenged with a protein antigen (see abstract). Maliszewski's method is not the same as that presently claimed in the instant application.

The application under examination does not activate dendritic cells or challenge with antigen. The claims in question relate to administration of Flt3L which leads to induction of tolerance and prevention of onset of an autoimmune disease, i.e. the subject does not have an existing disease. The application does not claim an increase in an immune response, it claims the opposite, a tolerogenic response. Accordingly, the application under examination cannot be anticipated by Maliszewski and reconsideration and withdrawal of the rejection of Claims 1-3 and 7-9 under 35 U.S.C. §102(b) is respectfully requested.

Claims 1-12 have been rejected as allegedly lacking an enabling disclosure under 35 U.S.C. §112, first paragraph. The Examiner has cited the factors of *In re Wands* to challenge the enablement of the pending claims contending that the genus of autoimmune diseases which can be prevented by the methods claimed is not fully enabled. In the first instance and for the benefit of the record, Applicants respectfully submit that at the time of the filing of the present application, the ordinary skilled artisan practicing in this field readily accepted that:

1. dendritic cells were crucial regulators of immune responses in general;

2. the immature state of dendritic cells promotes immune tolerance in general; and

3. the CD8+ dendritic cells had been shown to be critical for maintaining tolerance to tissue-derived antigens.

The background of the specification indicates that the current view at the time of filing the application was that immature dendritic cells can induce tolerance.

Thus, the skilled person would have accepted that in view of Flt3L administration delaying or preventing the onset of diabetes in the NOD model, that delaying or preventing the onset of autoimmune conditions in general could be achieved without undue experimentation, a feat achieved for the first time, by the present invention. Applicants have attached two references which confirm this. See, Steinman and Nussenzweign (in particular from page 353) (Exhibit 1) and Heath and Carbone (e.g. Figure 4 legend) (Exhibit 2).

Guidance and Working Examples

On page 7, 2nd paragraph to page 8, end of the 1st paragraph of the Office Action, the Examiner has alleged that the application has not prevented diabetes. Applicants respectfully submit that the working examples in the instant application detail a carefully designed set of scientific experiments which not only indicate which subsets of dendritic cells are important in immune tolerance but show that with repeated Flt3L treatments at intervals, **prevention** of the onset of diabetes is achieved (e.g Example 12). Example 12 (last sentence) clearly states that repeated 10 day treatments prevented diabetes from developing. The other examples where the incidence of diabetes is reduced have different treatment regimes, i.e. single treatment regimes. Given the explicit support for a method of prevention, Applicants should not have to search for different verbiage based on the Examiner's unsupported and unclear contention concerning the pending claims. Specifically, the Examiner contends that the claims are directed "to prevention of an autoimmune disease and not the lack of blood sugar".

The Examiner's point with respect to a "lack of blood sugar", is not clear. The blood sugar readings are specific to the examples of the specification and a determination of the occurrence of diabetes (Applicants note that diabetes mellitus is characterized by an abnormality of glucose metabolism, which causes elevated glucose levels in both the blood and the urine).

With respect to the objection that there is unpredictability in making and using the invention of Claims 4 to 6, the Examiner cites Rifkin et al. as prior art (page 9, line 2 of the Office Action). Rifkin et al. was published after the date of filing of the instant application and is not, therefore, prior art.

State of the Art and Quantity of Experimentation

The Examiner cites a 2006 web page which states that currently there is no way to prevent Type I diabetes. Applicants respectfully submit that, the NOD mouse is a well-accepted model of diabetes and the instant application clearly demonstrates for the first time that a Flt3L treatment regime prevents the onset of diabetes in the model (please refer to Example 12).

With respect to Claims 9 and 10, the important factor in the prevention of disease as shown in Example 12, is the timing of administration not the species of Flt3L (mouse Flt3L was used). As indicated in Example 15, human Flt3L is more effective in expanding dendritic cells (2nd paragraph) but the repeated administration regime was not used. The Examiner cannot, therefore, conclude that there is variation in results as the experiments are not the same.

Accordingly, the clear teaching of the present invention coupled with explicit working examples in a representative species of the claimed genus fully enables the pending claims in complete satisfaction of the requirements under 35 U.S.C. §112, first paragraph. Thus, the rejection of claims 1-12 under 35 U.S.C. §112, first paragraph is overcome and withdrawal thereof is respectfully requested.

Based on the foregoing amendments and remarks the application is in condition for allowance which action is earnestly solicited.

Respectfully submitted,

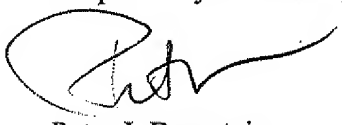

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EXHIBIT 1

Avoiding horror autotoxicus: The importance of dendritic cells in peripheral T cell tolerance

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Contributed by Ralph Marvin Steinman, November 13, 2001

The immune system generally avoids horror autotoxicus or autoimmunity, an attack against the body's own constituents. This avoidance requires that self-reactive T cells be actively silenced or tolerized. We propose that dendritic cells (DCs) play a critical role in establishing tolerance, especially in the periphery, after functioning T cells have been produced in the thymus. In the steady state, meaning in the absence of acute infection and inflammation, DCs are in an immature state and not fully differentiated to carry out their known roles as inducers of immunity. Nevertheless, immature DCs continuously circulate through tissues and into lymphoid organs, capturing self antigens as well as innocuous environmental proteins. Recent experiments have provided direct evidence that antigen-loaded immature DCs silence T cells either by deleting them or by expanding regulatory T cells. This capacity of DCs to induce peripheral tolerance can work in two opposing ways in the context of infection. In acute infection, a beneficial effect should occur. The immune system would overcome the risk of developing autoimmunity and chronic inflammation if, before infection, tolerance were induced to innocuous environmental proteins as well as self antigens captured from dying infected cells. For chronic or persistent pathogens, a second but dire potential could take place. Continuous presentation of a pathogen by immature DCs, HIV-1 for example, may lead to tolerance and active evasion of protective immunity. The function of DCs in defining immunologic self provides a new focus for the study of autoimmunity and chronic immune-based diseases.

The experiments of Paul Ehrlich at the turn of the last century helped establish the science of immunology. In addition to his prescient findings on specific immune receptors (1), Ehrlich used a collection of stains to identify many types of white blood cells, including lymphocytes, the mediators of immunity. Ehrlich's experiments on antibodies led him to conclude that immunity is exclusively directed to foreign materials or antigens; normally there is no reactivity or tolerance to self. For example, he found that a goat made antibodies to red blood cells from other goats but not to its own red blood cells. Thus the body avoids an immune attack on itself. He states: "We pointed out that the organism possesses certain contrivances by means of which the immunity reaction, so easily produced (induced) by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins . . . so that one might be justified in speaking of a 'horror autotoxicus' of the organism" (p. 253, ref. 2). Actually, autoimmunity does develop in many diseases, including systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, psoriasis, and juvenile diabetes.

Ehrlich suggested that self-reactive lymphocytes could be silenced or tolerized by losing their self-specific receptors (p. 208, ref. 2), a prediction that has proven correct for antibody-producing B cells (3, 4). Here we propose that one type of white cell, the dendritic cell (DC), has major roles in silencing self-reactive T lymphocytes. These T cells are produced centrally in the thymus, where some self-reactive T cells are tolerized through the aegis of thymic DCs and other antigen-presenting cells (Fig. 1). Then T cells emerge into the periphery to patrol and defend the body against pathogens. Here evidence will be outlined that DCs silence peripheral T cells as well (Fig. 1). Before going over this information, two background topics need to be considered: the limitations of central thymic tolerance and the traditional function of DCs in inducing immunity to foreign antigens especially infections (5, 6).

Central Tolerance

The Importance of Central Tolerance in Preventing Autoimmunity. So-called central tolerance is the best-known pathway to silencing self-reactive lymphocytes. Developing B and T cells rearrange Ig and T cell receptor genes to produce clones of lymphocytes with unique antigen receptors. Gene rearrangement is random, so that both self- and nonself-reactive clones are produced in the central lymphoid organs, the bone marrow, and thymus. However, when self antigens are present during development, the autoreactive B and T cells or their receptors can be selectively deleted as envisaged by Burnet (7) and Lederberg (8). Owen first uncovered experimental evidence for this fundamental developmental route to tolerance in his studies on cattle (9). He observed that fraternal twins, although genetically different, failed to mount immune responses to each other's cells. This finding was remarkable in view of Ehrlich's observations that any individual reliably formed antibodies to the cells of another individual. The basis for the tolerance in cattle twins was a shared placental circulation, whereby the twins became hematopoietic chimeras during development. Billingham, Brent, and Medawar exploited Owen's finding when they made the dramatic discovery that an injection of foreign white blood cells into neonatal mice could induce tolerance to transplantation antigens, especially products of the foreign major histocompatibility complex (MHC) (10). The underlying mechanisms for central tolerance of developing lymphocytes were then appreciated once methods were developed to identify these lymphocytes. It was noted that self-reactive T and B cells were deleted centrally (11–14) or, in the case of B cells, their receptors could be edited and replaced by receptors for foreign antigens (3, 4).

DCs and Central Tolerance. DCs play an important role in the self/nonself distinction imposed by the thymus. Located almost entirely in circumscribed medullary regions (15, 16), DCs present self antigens to developing T cells and delete lymphocytes with autoreactivity (17–20) (Fig. 1).

Limitations of Central Tolerance. Despite its effectiveness for some classes of antigens, central tolerance has major limitations (Table 1). Self-reactive T and B cells can escape deletion and editing (21) or, as Nossal vividly summarized, "The immunological self exerts its purgative mastery on lymphocytes only to

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Abbreviations: DC, dendritic cell; MHC, major histocompatibility complex; TLR, toll-like receptors; TNF, tumor necrosis factor; IFN, interferon.

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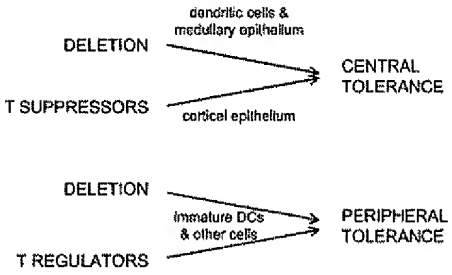

Fig. 1. Central and peripheral mechanisms for avoiding horror autotoxicus via T lymphocytes. In the thymus (central tolerance) and in other parts of the body (peripheral tolerance), self-reactive T cells can either be eliminated (deleted) or regulated (suppressed) by other T cells. Several types of antigen-presenting cells can bring about tolerance as shown by the arrows. DCs play a pervasive role, particularly for dying cells and innocuous self and environmental proteins that have to be captured and processed before presentation (as MHC class I and II–peptide complexes) to antigen receptors on T cells.

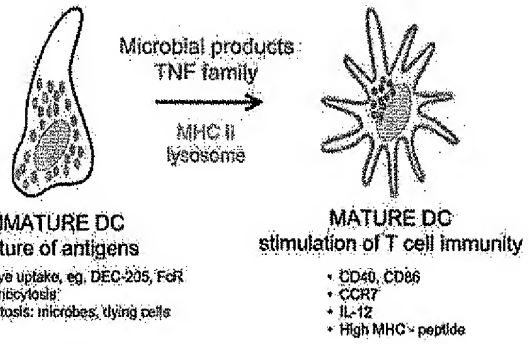

Fig. 2. DC maturation, a control point for regulating tolerance and immunity. Immature DCs capture antigens by several pathways, whereas mature DCs stimulate T cell immunity, i.e., helper and cytolytic effector lymphocytes as well as memory. Maturation stimuli act via TLRs (whereln distinct microbial products act although distinct TLR) and TNF family receptors (such as TNF itself and CD40L). Maturation leads to several changes, including: the redistribution of MHC class II molecules and MHC–peptide complexes from within the endocytic system to the cell surface as diagrammed here, the production of several cytokines and membrane associated T cell stimulatory molecules, and the remodeling of expressed chemokine receptors.

a degree" (22). Many self antigens may not access the thymus (23), whereas others are expressed later in life, after the lymphocyte repertoire has been formed (24). Furthermore, the body is constantly exposed to innocuous nonpathogenic environmental antigens to which it remains tolerant, e.g., proteins and commensal organisms within our airways and intestines. The chance that lymphocyte receptors for foreign antigens crossreact with self proteins is also substantial (25). These limitations of central tolerance necessitate effective peripheral silencing mechanisms (26, 27). Indeed, T lymphocytes can be tolerized in peripheral tissues (28). Here we propose that DCs function to control antigen-specific peripheral tolerance (Fig. 2), which may seem counterintuitive, because DCs have many critical roles in inducing immunity. We will propose that the tolerizing function of DCs occurs in the steady state, i.e., before an acute infection, and is essential to their subsequent function in generating antimicrobial immunity.

DC Maturation: The Risk of Autoimmunity and Chronic Inflammation During the Defense Against Pathogens

DC Maturation as a Control Point for Initiating Immunity. DCs are specialized to process antigens, presenting them as peptides bound to MHC products and initiating immunity. However, the capture of antigens and the initiation of immune responses are distinct functions carried out by DCs at different stages of development, termed immature and mature (Fig. 2). These terms have some imprecision (see *Questions*), because they encompass cells found in different organs and pathologic settings as well as DC subsets and DCs generated in culture by different methods. Nevertheless, most types of immature DCs are known to capture antigens, both soluble and particulate, and have a number of receptors and intracellular compartments appropriate for the task (29). During maturation, additional functions develop that enhance the ability of DCs to induce immunity (Fig. 2) (30–41). Some changes that take place on maturation and enhance immunogenicity include: (*i*) increased formation of stable MHC–peptide complexes (36, 42–44); (*ii*) higher expression of membrane molecules like CD86 and other B7 family members for T cell binding and activation (45–47); (*iii*) new synthesis of cytokines that influence T cell proliferation and differentiation (48, 49); and (*iv*) altered production of chemokines and chemokine receptors that intensify movement of DCs into lymphatic vessels and lymphoid organs (50–53).

Janeway has independently emphasized a theme that parallels DC maturation (54, 55). He reasoned that a key component to immunogenicity, distinct from antigen processing, is the capacity of pathogens to activate antigen-presenting cells through pattern recognition receptors. These receptors induce expression of costimulatory functions required for immunity. The changes associated with pathogen recognition are encompassed by the events of DC maturation. Yet DC maturation also occurs in the absence of infection, during such powerful T cell immune responses as transplantation (56), contact allergy (57), and autoimmunity (58).

Table 1. The limitations of central tolerance in avoiding horror autotoxicus

Self-reactive lymphocytes escape negative selection.
Certain self antigens may not gain sufficient access to thymic antigen-presenting cells.
Many self antigens are expressed only after the T cell repertoire has been formed.
Many innocuous environmental proteins enter the body postnatally.
Lymphocyte receptors for foreign antigens can crossreact with self.

The Risks of DC Maturation. To initiate T cell immunity to pathogens, DCs must accomplish two things: process the pathogen to form MHC–peptide complexes (antigen presentation) and differentiate or mature as summarized above. However, maturation creates a problem with respect to self/nonself discrimination. Consider influenza infection of the lung as an example: DCs not only capture the virus but also are likely to be taking up dying influenza-infected cells (59, 60). Furthermore, DCs capture airway proteins continuously, even without the provocation of a pathogenic infection (61, 62) (Fig. 3). As Ehrlich would have predicted, the development of autoimmunity is the exception, not the rule, during recovery from respiratory and other infections. He pointed out: "During the individual's life, even under physiological although especially under pathological conditions, the absorption of all material of its own body can and must occur very frequently. The formation of tissue autotoxins would therefore constitute a danger threatening the organism more frequently and much more severely than all exogenous injuries" (p. 253, ref. 2). Even during the influenza pandemic of World War I, most infected people recovered without residual chronic reactivity to their airways or airway proteins. How, then, do DCs stimulate immunity to influenza but at the same time avoid

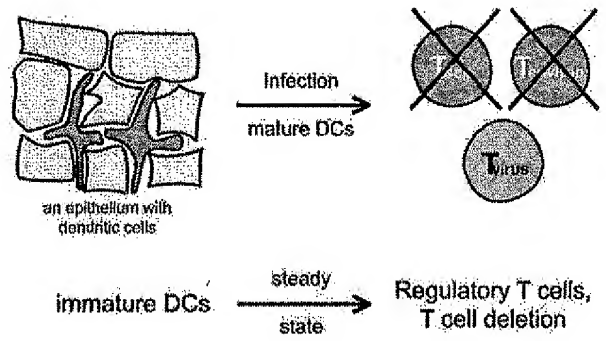

Fig. 3. Overcoming the risk of autoimmunity and horror autotoxicus inherent to the maturation of DCs on exposure to pathogens. During infection, DCs mature, e.g., in response to pathogen signals via TLRs (Fig. 2). However, the maturing DCs will likely be presenting peptides not only from the pathogen but also from dying self tissue and innocuous environmental proteins. To overcome this risk, it is proposed that immature DCs induce antigen-specific peripheral tolerance in the steady state, before DC maturation during inflammation and infection. DCs can do so by deleting naïve T cells or inducing regulatory T cells. The tolerized T cells can either be self-reactive lymphocytes that have escaped central tolerance or T cells reactive to innocuous proteins in the environment.

stimulation of T cells reactive to self and innocuous environmental antigens?

A Proposal: Immature DCs in the Steady State Define Immunologic Self and Tolerize T Cells Peripherally, Avoiding the Risks Associated with DC Maturation During Infection. We suggest that DCs in the steady state, before infection or inflammation, critically define immunologic self and prevent the induction of both autoimmunity and chronic inflammation against environmental proteins (Fig. 3). According to this theory, proteins captured and processed by DCs in the steady state are tolerogenic, i.e., the DCs silence the corresponding antigen-specific T cells. As a result, when the same proteins are presented during infection, the immune response is able to focus on the pathogen, not on self or environmental antigens that are presented along with the pathogen (Fig. 3). Reciprocally, chronic inflammatory diseases against otherwise nonpathogenic antigens would be directed primarily to proteins that are not presented by DCs in the steady state.

Immature DC Function in the Steady State: Migration and Uptake of Self and Environmental Proteins

The Distribution, Migration, and Turnover of DCs in the Steady State. DCs are located at body surfaces, especially the skin (30) and airways (61, 63), in the interstitial spaces of many organs (64), lymphoid tissues (65, 66), blood (67), and, importantly, afferent lymphatics (68–71), the conduits between peripheral tissues and immunologically active lymph nodes. DCs can insinuate themselves into epithelia (61, 72), possibly after the interaction of CCR6 receptors on DCs (73) with epithelial MIP-3α/CCL20 (74). By expressing important molecular components of intercellular junctions, DCs at body surfaces may even insinuate through tight epithelia, extending their processes into the environment to capture proteins without breaking the epithelial barrier; this phenomenon can be enhanced by microbial stimuli (75). In mucosal associated lymphoid tissues, DCs lie beneath the antigen-transporting epithelia, again in the perfect niche to capture antigens transported through epithelial M cells (76).

In the steady state, immature DCs circulate between nonlymphoid and lymphoid tissues at a rapid rate. At least some, and perhaps most, peripheral DCs enter afferent lymphatics and then migrate to the T cell area, where they die, because few DCs are present in efferent lymphatics that leave the lymph node (70, 77). In mice, the life span of most DCs in the lung and lymphoid tissues is <2 days (78–81). DC migration from epithelial surfaces and deeper tissues to lymphoid organs can be further increased by applying a contact allergen (68) or by administering inflammatory cytokines or microbial products (82, 83). Another group of DCs, termed plasmacytoid cells (84, 85), enter the lymphoid tissues directly from the blood (86). Thus, DCs patrol most tissues continuously in the steady state. They are perfectly positioned to capture self and environmental antigens and to access the corresponding specific T cells (reviewed in ref. 87).

DCs Capture Antigens in the Steady State. The proposal under consideration here is that immature DCs in the steady state are vital to defining self in the periphery. Appropriately, DCs efficiently pick up and process proteins, e.g., from the airway (61, 62), blood (88–90), muscle (71), and intestine (91). The experimental approach is to inject the antigen without any other stimulus or adjuvant, isolate DCs a day later, and then test whether the DCs can present the antigen to specific T cells in culture. In every case, DCs show high levels of antigen presentation to specific T cells, whereas other cells exhibit little if any activity (89). Likewise DCs continually capture particulates, including dying cells *in vivo*. DCs that traffic through the liver and into hepatic lymphatics can pick up latex particles and colloidal carbon (92), Langerhans cells in skin-draining lymph nodes contain melanin granules acquired from cells in the skin (93), and DCs capture intestinal epithelial cells before entry into the mesenteric lymphatics (94). Thus, the normal process of cellular turnover in nonlymphoid tissues appears to provide circulating DCs with a constant supply of self antigens for processing and presentation (94, 95), an important prerequisite for peripheral tolerance (96). Together, efficient antigen capture, rapid turnover, and widespread circulation through tissues allow DCs to perpetually sample self and environmental antigens.

Two New Lines of Evidence for Peripheral Tolerance via Immature DCs

Peripheral T Cell Deletion via Immature DCs. Although antigen uptake by DCs in the steady state is well documented *in vivo* (above), the immunologic consequences have not been pursued. It turns out that peripheral tolerance can ensue. One mechanism involves deletion of specific T cells, a consequence that parallels central or thymic tolerance. A recent experiment, which revealed this role of DCs *in situ*, involved the targeting of antigens to DCs through an adsorptive endocytosis receptor, DEC-205 (97, 98). This receptor is abundantly expressed on many DCs in the T cell areas of peripheral lymphoid tissues, i.e., in the ideal place to present captured antigens to T cells circulating through lymphoid organs (99). We chose DEC-205 for targeting antigens to DCs, because it mediates uptake of bound ligand, and its cytosolic domain contains an EDE triacidic amino acid targeting sequence that delivers ligands to MHC class II containing compartments 30–100 times more effectively than homologous receptors (100). The proteins delivered by DEC-205 to such compartments are processed and loaded onto MHC class II molecules (100). Because natural ligands for DEC-205 are not yet known, anti-DEC-205 antibodies were engineered to carry antigenic peptides from a model antigen hen egg lysozyme (HEL). The anti-DEC/HEL antibody, in fact, targeted selectively to DCs *in situ* in the steady state, and when corresponding TCR transgenic T cells were exposed to these targeted DCs, the T cells proliferated vigorously at first (98). Within a week, however, the majority of the responding T cells were deleted, and the mice became tolerant, unable to be primed by injection of

peptide with the powerful Freund's adjuvant. This peripheral tolerance could be converted to immunity if the anti-DEC-205/HEL were given together with a DC maturation stimulus.

In these experiments, the doses of injected protein antigen were low (<1 μgm of antibody or <15 ngm of peptide), and the dose of antigen-specific T cells high (2×10^6 were tolerized; the total number of T cells in a mouse is estimated to be $\approx 2 \times 10^8$, of which $< 2 \times 10^4$ typically respond to any one antigen). Therefore, through the use of DEC-205 to target DCs in the steady state, small amounts of an intact protein can lead to either tolerance or immunity. These results contrast with the prior literature on peripheral tolerance where much higher doses of preprocessed peptides (100 μgm or more) have been used (101, 102). The tolerance observed after targeting of proteins to DCs *in situ* also bears on striking observations that bone marrow-derived cells—presumably DCs (103)—can mediate either peripheral deletion (104, 105) or anergy (106, 107) *in situ*. The DEC-205 targeting experiments specifically implicate DCs as the inducers of peripheral tolerance by T cell deletion. This outcome can be converted to immunity if the DCs additionally receive an appropriate maturation stimulus.

Induction of Regulatory T Cells by Immature DCs. The induction of regulatory T cells by DCs is another mechanism for peripheral tolerance. There may be different types of regulatory or suppressor T cells, e.g., those formed in the thymus and in the periphery. Regulatory cells are found as a small fraction (<5%) of the T cells in blood, and they are able to suppress the responses of other T cells to powerful stimuli (108–112). Because these regulatory cells dampen the responses of other effector (helper and killer) lymphocytes, they give rise to functional tolerance. The regulatory mechanisms are at this time unclear but, in addition, how are these cells induced in the first place?

The concept is that immature DCs are responsible for the formation of peripheral regulatory T cells, which has emerged during studies with DCs in humans. The approach is to isolate precursors from blood [either CD34 + proliferating progenitors (113) or CD14 + nonproliferating monocytes (39, 40)] and convert these to DCs *ex vivo* before reinfusion. The field is still in its early stages (reviewed in refs. 114 and 115), but one of the goals is to use DCs as "nature's adjuvant" to immunize patients against antigens in their tumors. The *ex vivo* approach is valuable, because DCs can be loaded with large arrays of antigens, including those expressed by tumor cells (116, 117), and because DC maturation can be regulated.

The initial studies of humans *in situ* were carried out with mature DCs and a model MHC (HLA-A2.1) binding influenza viral peptide. A single injection of peptide-charged DCs rapidly expanded peptide specific immunity an average of 5-fold (118), whereas a booster dose enhanced T cell functional affinity 30- to 100-fold (119). In contrast, when immature peptide-pulsed DCs were injected, influenza-specific CD8 + interferon (IFN)γ-secreting T cells virtually disappeared from the blood stream; in their place, peptide-specific IL-10-secreting T cells appeared (41). At least some regulatory T cells are known to produce high levels of IL-10 (120, 121). When tested, the peptide-specific T cells induced by immature DCs were indeed able to suppress the effector function of IFNγ-secreting cells (M. V. Dhodapkar and R.M.S., unpublished work). The induction of regulatory cells was transient and reversed within 1–3 months, with a return of the IFNγ-secreting T cells. These *in vivo* experiments in humans, coupled with additional studies in tissue culture (122), demonstrate the capacity of immature DCs to rapidly induce regulatory T cells. The latter, it is known, are able to silence effector T cells including autoaggressive ones in mice (27, 110, 123–127). Possibly regulatory T cells operate by changing the function of DCs (128). In any case, the induction of these suppressive T cells provides another mechanism whereby DCs could induce antigen-specific peripheral tolerance.

In summary, we propose that immature DCs define immunologic self, silencing the T cell repertoire to self and environmental antigens captured during the steady state. When the DCs subsequently mature in response to infection, the preexisiting tolerance nullifies the development of reactivity to innocuous antigens and focuses the immune response on the pathogen. In the thymus, DCs delete self-reactive T cell clones, whereas in the periphery, DCs delete T cells and induce the formation of regulatory T cells. Our proposal draws on the known migratory and antigen-capturing activities of immature DCs in the steady state and is supported by recent evidence that DCs tolerize *in situ*.

Questions and Challenges That Arise from This Concept of Peripheral Tolerance

How Does the Idea that DCs Control Tolerance Differ from Other Theories? Mechanisms of peripheral tolerance have relied on what is termed the two-signal notion of acquired immunity (reviewed in ref. 129). The proposal is that the presentation of self antigens or "signal one," in the absence of costimulation, or "signal two," induces T cell anergy or deletion. However, it is difficult to tolerize an animal with antigen alone, i.e., by the injection of intact proteins or even preprocessed peptides (101, 102), possibly because antigens need to be captured in sufficient amounts by immature DCs in order for tolerance to ensue. There also is information that antigens on non-DCs are ignored and not truly tolerogenic (130, 131). In other instances, antigens expressed by non-DCs are able to tolerize but only after processing by bone marrow derived cells, possibly DCs (105, 132). The "signal one" theory of tolerance therefore seems to be oversimplified and suffers from a dearth of evidence with intact soluble and cell-associated proteins *in vivo*.

We are instead proposing that the MHC peptide complexes produced by antigen processing become effective tolerogens when presented by DCs in the steady state. Also, the induction of tolerance by immature DCs likely requires a number of special features of these cells, not just "signal one". Already evident are: (*i*) the efficient capture of antigens, including the exogenous pathway whereby DCs are specialized to form MHC class I-peptide complexes from soluble proteins, immune complexes, and dying cells (reviewed in ref. 29); (*ii*) the potential to bind T cells to be tolerized via receptors like DC-SIGN, a newly recognized lectin that interacts with intercellular adhesion molecule-3 on resting T cells (133); (*iii*) the production of IL-10 and possibly other regulatory cytokines (see below); and (*iv*) the ability to migrate to positions that optimizes access to antigens and T cells *in situ*. Likewise, the alterations that convert DCs to the immunogenic state are beginning to be unraveled. On microbial challenge, it is known that maturing DCs: (*i*) secrete cytokines like IL-12 that cause the differentiation of T cells to IFNγ–producing effectors (134); (*ii*) express increased levels of the CD80 and CD86 costimulatory molecules (45, 46), particularly in coclusters with MHC–peptide complexes (36); and (*iii*) regulate other costimulatory B7 family members, e.g., a molecule called B7-DC is induced (47). Despite progress in this area, there is a great deal to be learned about the features of DCs that regulate the balance between T cell immunity and tolerance.

Are There Different Types of Immature DCs? There is not simply one discrete immature and mature type of DCs. Instead, there is a differentiation pathway triggered by a spectrum of external stimuli (microbial products, members of the tumor necrosis factor (TNF) family, other cytokines, heat shock proteins), possibly with distinct outcomes. In addition there are subsets of immature DCs, which can differ in their receptors for antigen

uptake, the cytokines produced on stimulation, and the microbial products to be recognized (135). Currently, a perplexing area is the relationship between immature DCs produced in tissue culture and the tolerizing, DEC-205 positive, DCs within peripheral lymphoid organs. Immature DCs developing in culture go through a stage where antigens are captured, but MHC class II–peptide formation is weak (44). In contrast, the immature DCs in peripheral lymphoid organs (targeted with our anti-DEC antibodies, above) efficiently process and present antigens to induce tolerance (98, 136).

The precise physiologic counterpart of the frequently studied immature DC, produced with cytokines in culture, is not yet obvious. It may be the Langerhans cell and its homologues in other surface epithelia and/or the monocyte-derived DCs migrating from tissues after encountering antigen (137) or lymphatic endothelial cells (138). Understanding the relationships between these DCs is important, because different immature cells may induce peripheral tolerance by distinct mechanisms, such as deletion and induction of regulatory T cells.

Another key variable may be the capacity of immature DCs to produce IL-10 or other suppressive cytokines like transforming growth factor β. MHC class II bearing, IL-10 producing cells can regulate experimental autoimmune encephalomyelitis in mice (139). High amounts of IL-10 are made by DCs isolated from lung (140) and intestine (141) and by DCs developing *ex vivo* from monocytes (142–144). This IL-10 may lead to tolerance in several ways: IL-10 can itself suppress T cells (145); IL-10 may be required to differentiate regulatory T cells (125, 146); or IL-10 can act on DCs to decrease their function (147, 148) or make them tolerogenic (149). In contrast, DCs in the T cell areas of lymphoid tissue are not yet known to be producing IL-10 in the steady state. These DCs nonetheless can efficiently form MHC class II–peptide complexes and delete T cells in the steady state (98), but they lack other features of maturing DCs, such as IL-12 production and high levels of CD86 and CD40 (150, 151).

What Controls DC Maturation? Microbial signaling through toll-like receptors (TLRs) is an effective way to mature DCs to their immunogenic state (150–153). However, DC maturation can also be induced under sterile circumstances, as in the cases of transplantation (56) and contact allergy (154). In these intense T cell-mediated immune responses, the requisite receptors for DC maturation have yet to be identified. A recent proposal is that TLRs are engaged by endogenous ligands, such as heparin sulfates and hyaluronans (155, 156). Many cell types produce cytokines when signaled through TLRs and the associated MyD88 adaptor protein (157). However, in DCs there is an additional MyD88 independent TLR-dependent pathway that leads to maturation and the capacity to initiate immunity (158, 159). TNF family members, e.g., CD40L on mast cells and platelets, and hematopoietin families, e.g., granulocyte macrophage–colony-stimulating factor, IL-4, IL-13, additionally influence DC development and maturation. These non-TLR stimuli may produce DCs with different functions or, alternatively, they may be required in concert with TLR signaling for full DC activity. For example, DCs require both a microbial and a TNF family stimulus to make large amounts of IL-12, a key cytokine for strong cell-mediated immunity (160). Increased understanding of DC maturation should yield new ways to manipulate this critical control point in immunity and tolerance.

Do Other Antigen-Presenting Cells Contribute to the Induction of Tolerance? Other cells can contribute to tolerance in important ways (Fig. 1). Thymic medullary epithelial cells can induce central tolerance (161–163) (reviewed in ref. 164), possibly to epithelial and neuroendocrine antigens that they synthesize and that are not otherwise available to thymic DCs. Thymic cortical epithelial cells recently have been shown to induce suppressor T cells (165, 166), which seem related functionally to the peripheral regulatory T cells induced by immature DCs (reviewed in refs. 126, 127, and 167). Liver sinusoidal endothelial cells also can silence antigen reactive T cells, perhaps those specific for intestinal proteins that continually enter the portal circulation (168). B cells have been implicated in T cell tolerance, but, in the case of a B cell lymphoma, tolerance is induced only after the B cells are processed by other bone marrow-derived cells, possibly DCs (107). At this time, DCs cannot be regarded as exclusive mediators of tolerance or as exclusive mediators of immunity. Instead, DCs are specialized and efficient controllers of immunity, particularly when proteins (self, environmental, pathogenic) must be internalized and processed before presentation to quiescent peripheral T cells.

Implications

Monitoring and Manipulating Tolerance at the Level of Antigen-Presenting DCs. Dozens of chronic inflammatory diseases are considered to be autoimmune in origin, and several autoantigens are known (169). When initiated, autoimmunity can selectively destroy tissue targets. For example, in juvenile diabetes, T cells attack insulin-producing cells in the pancreatic islets of Langerhans, and in multiple sclerosis, T cells attack the glial elements of the central nervous system. Other chronic inflammatory diseases may represent a failure of tolerance mechanisms toward normally innocuous environmental proteins and microorganisms rather than self. Inflammatory bowel disease, for example, may be directed to nonpathogenic bacteria in the intestine.

We have reasoned (Fig. 3) that the function of DCs in tolerance is most important for those self and environmental proteins that can be processed during an infection. Other self antigens could evade DC-mediated peripheral tolerance because of a low level of expression in the steady state or poor access to DCs (105, 132). The antigens that are not efficiently presented by DCs in the steady state might be good candidates to elicit autoimmune diseases. If these proteins begin to be processed *de novo* under conditions compatible with DC maturation, e.g., during an infection when proteases are released by inflammatory cells or from microbes themselves, the previously ignored self proteins may be presented by mature DCs and autoimmunity could ensue (170, 171).

The standard experimental and therapeutic approaches to the induction of tolerance are to use antigen-nonspecific agents, which impede the function of all T cells, or T cells responding to any antigen. As in the case of protective immunity, DC biology opens up the possibility for antigen-specific monitoring and manipulation of autoimmunity. Mature DCs might be used experimentally to identify disease-producing autoantigens, as recently shown for an autoimmune disease called primary biliary cirrhosis (172), whereas immature DCs might be used to dampen the autoimmune response in patients. One implication of our proposal is that the targeting of antigens to DCs in specific states of maturation may provide novel strategies for vaccination and immune therapies (41, 98, 173) (M. V. Dhodapkar and R.M.S., unpublished work).

Tolerance Induction by Persistent Pathogens. The most challenging aspect of DC-induced peripheral tolerance relates to persistent pathogens, both infectious agents and tumors, which are captured by DCs. Some persistent infections, like herpes simplex virus (174), cytomegalovirus (175), and plasmodium falciparum (176), may inhibit DC maturation and decrease the efficacy of the host immune response. We would like to propose that an additional strategy on the part of the pathogen is to actively

induce tolerance by virtue of continuous capture and presentation by immature DCs.

In the case of HIV, a very large number of virions are produced continuously in infected individuals (177, 178). Tissue culture experiments indicate that DCs can drive the replication of virus in T cells (Fig. 4 *Upper*) (179, 180). DCs can either replicate HIV, which then infects T cells in large numbers, or simply capture and directly transmit HIV to permissive T cells. During chronic infection, patients are essentially asymptomatic. Their DCs may well be in an immature or steady state and may take up virions in sizable quantities. Furthermore, immature DCs express several HIV receptors, such as CD4, CCR5, and DC-SIGN (181–184), and support virus replication (182, 185). HIV may therefore exploit immature DCs in an immunologic sense and not just a virologic one (Fig. 4 *Lower*). The virus becomes a very efficient form of "self," possibly inducing regulatory T cells and/or deleting HIV reactive T cells from the repertoire. Other chronic infections may likewise induce regulatory T cells (186). However, in the case of HIV, the amount of antigen and the targeting of virus to receptors on immature DCs should intensify immune evasion through the induction of tolerance. Many serious infections, such as tuberculosis and influenza, can be lethal, but the immune system assists the majority of infected individuals (>90%) in recovering without residual disease. Yet in HIV infection, the immune system is unable to defeat the pathogen in the vast majority of people, consistent with some built-in restraint mechanism such as tolerance. The theory proposed here may help to explain this ominous property of the AIDS epidemic.

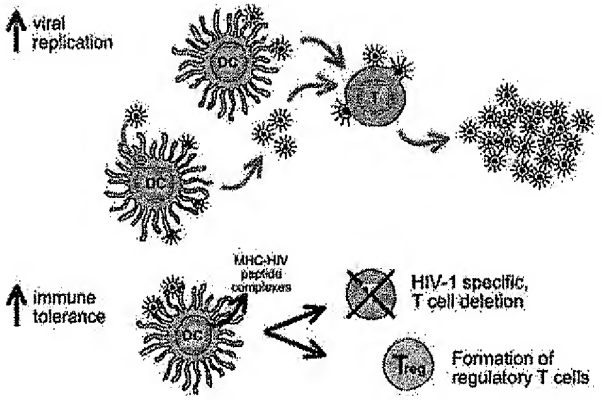

Fig. 4. Potential sites for involvement of DCs in HIV pathogenesis. In the virologic pathway (*Upper*), emphasized in the past, DCs catalyze HIV replication in T cells. In the immunologic pathway (*Lower*) proposed here, immature DCs continually capture and even replicate HIV virions, which induces peripheral tolerance, including regulatory T cells, thereby blocking the effector or protective limbs of the immune response.

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EXHIBIT 2

CROSS-PRESENTATION IN VIRAL IMMUNITY AND SELF-TOLERANCE

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T lymphocytes recognize peptide antigens presented by class I and class II molecules encoded by the major histocompatibility complex (MHC). Classical antigen-presentation studies showed that MHC class I molecules present peptides derived from proteins synthesized within the cell, whereas MHC class II molecules present exogenous proteins captured from the environment. Emerging evidence indicates, however, that dendritic cells have a specialized capacity to process exogenous antigens into the MHC class I pathway. This function, known as cross-presentation, provides the immune system with an important mechanism for generating immunity to viruses and tolerance to self.

ISOTYPE SWITCHING
When B cells change their class of antibody (immunoglobulin) production from one isotype to another, for example from IgM to IgG.

THYMIC SELECTION
The process of choosing which thymocytes develop into mature T cells on the basis of the specificity of their T-cell receptors.

PERIPHERAL TOLERANCE
The generation of tolerance to self for mature T cells that have left the thymus and are recirculating in the periphery.

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T lymphocytes can be separated into two subpopulations on the basis of their expression of the cell-surface markers CD4 and CD8. The $CD4^+$ subset is primarily responsible for providing help to other immune cells through direct cell–cell interactions or the secretion of cytokines. Collaboration with B cells, for example, leads to ISOTYPE SWITCHING and enhanced antibody production. $CD4^+$ T cells also have an important role in the induction of inflammatory responses and the generation of $CD8^+$ T-cell immunity. Effective priming of $CD8^+$ T cells leads to their development into mature cytotoxic T lymphocytes (CTLs), which are best known for their capacity to kill virus-infected cells. In this review, for simplicity, we will refer to $CD4^+$ T cells as 'helper T cells' and $CD8^+$ T cells as 'CTLs'.

Helper T cells and CTLs use their T-cell receptors to recognize peptide antigens presented by molecules encoded by the MHC. Helper T cells recognize peptides presented by MHC class II molecules, whereas CTLs are restricted to MHC class I molecules. This preference for different classes of MHC molecules relates to a demarcation in the antigen-processing pathways that supply peptides. MHC class II molecules generally present peptides derived from exogenous antigens that enter the cell by the endocytic route, whereas MHC class I molecules present endogenously derived antigens, usually synthesized within the cell presenting the antigen (FIG. 1a,b). The targeting of CTLs to endogenously synthesized antigens is important as it ensures that virus-specific CTLs only kill cells that are directly infected with virus. Bystander cells that simply endocytose viral debris from infected neighbouring cells will not process this antigen into the MHC class I pathway and will therefore not be targeted by CTLs.

Although CTLs perform the very important function of killing cells infected with viruses or intracellular bacteria, their ability to destroy target tissues comes at a price. CTLs with specificity for self-antigens can sometimes attack normal host tissues and cause autoimmunity[1,2]. For this reason, it is very important to maintain tight control over the generation of effector CTLs, maximizing their pathogen-fighting capacity, while minimizing their autoimmune potential. Although this is primarily achieved during THYMIC SELECTION, in which most self-reactive T cells are deleted, other controls are important. Self-reactive CTLs can, for example, be regulated by PERIPHERAL-TOLERANCE mechanisms[3–7], and also by checkpoints that prevent their maturation in the absence of signals from helper T cells[8–10] (FIG. 2). In this review, we will examine the role of cross-presentation in the generation of CTL immunity (cross-priming) and in the maintenance of self-tolerance (cross-tolerance).

What is cross-presentation?

Naive T cells recirculate throughout the secondary lymphoid compartment, moving between the lymph

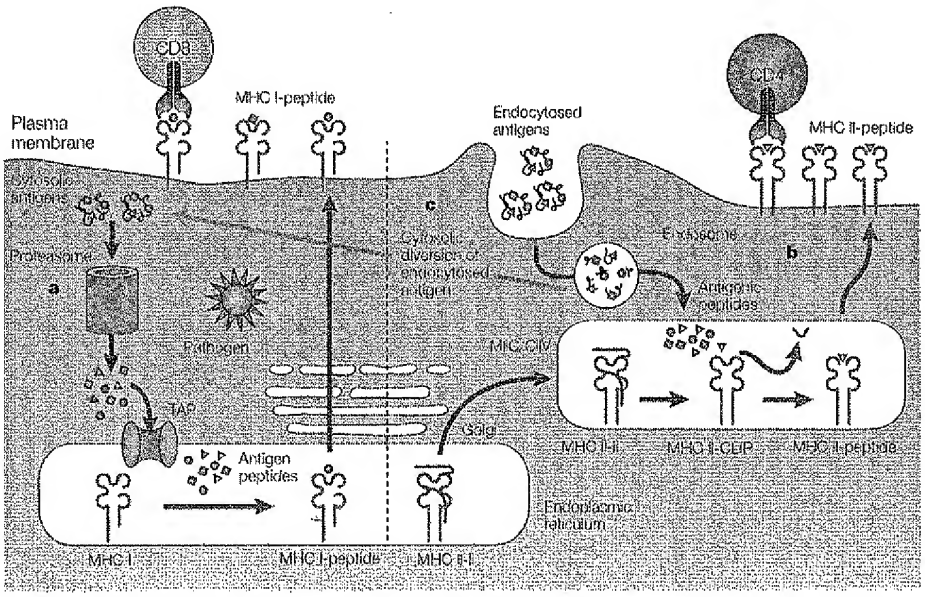

Figure 1 | **Different antigen-processing pathways for the MHC class I and class II molecules. a** | MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self or pathogen origin. These proteins are degraded into peptides by the proteasome and then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum for loading on MHC class I molecules. **b** | By contrast, MHC class II molecules present proteins that enter the cell through the endocytic route. During maturation of MHC class II molecules, they are prevented from binding to endogenous antigens in the endoplasmic reticulum by association with the invariant chain (Ii). Invariant chain–MHC class II complexes (MHC II–Ii) move through the Golgi to the MIIC/CIIV compartment where the invariant chain is degraded to CLIP (for class II-associated invariant-chain peptide). CLIP is then removed from the CLIP–MHC class II (MHC–CLIP) complexes and exchanged for antigenic peptide. **c** | Dendritic cells can endocytose antigens from other cells and cross-present them to $CD8^+$ cytotoxic T lymphocytes. The TAP-dependence of such cross-presentation[14,58], indicates that it involves diversion of the cellular antigens into the conventional MHC class I pathway, although the mechanism(s) for this diversion are as yet undefined. In most cases, these antigens will also be processed into the MHC class II presentation pathway for recognition by $CD4^+$ helper T cells. (MIIC, MHC II loading compartment; CIIV, MHC II vesicles.)

nodes, blood and spleen. This limited recirculation pattern means that many pathogens enter the body at sites where they will not directly encounter naive T cells. For their initial encounter with antigen, T cells rely on dendritic cells (DCs) to capture pathogen products from the site of infection and transport them to the draining lymph nodes. In this way, naive T cells can scan the entire body for the presence of pathogens simply by scanning antigens presented on DCs that migrate to the secondary lymphoid compartment. As well as transporting antigen, DCs express co-stimulatory molecules that allow them to activate naive T cells, classifying them as professional antigen-presenting cells (APCs). So, once a specific encounter occurs between a T cell and a DC, T cells are activated, proliferate and differentiate, and are then able to enter peripheral tissues to fight the invading pathogen.

For MHC class II-restricted responses, which are directed at exogenous antigens, it is easy to imagine how DCs can capture pathogen products and present them to MHC class II-restricted helper T cells in the draining lymph nodes. For MHC class I-restricted responses, which are generally thought to target antigens that are synthesized within the cell presenting the antigen, it becomes a little more complicated to describe the role of the DC. In the simplest case, DCs could themselves be infected with the pathogen, so allowing MHC class I-restricted presentation of pathogen-derived antigens. Not all pathogens are known to infect DCs, however, and pathogen-infected DCs are often functionally compromised[11–17]. Therefore, as suggested by Bevan some years ago[18], an exogenous pathway for processing MHC class I-restricted antigens within DCs might be necessary. In fact, Bevan's hypothesis came from his discovery of such a pathway for the priming of CTL immunity[19]. He showed that protein antigens (in this case minor histocompatibility antigens) that were synthesized in one cell could be captured as exogenous antigens by APCs, processed into the MHC class I antigen-presentation pathway, and used to prime CTL immunity. Bevan termed this 'cross-priming', and we have subsequently defined the antigen-processing associated with cross-priming as 'cross-presentation'. Cross-presentation has been previously used in two contexts in the literature. In the first case, it simply meant processing of exogenous antigens into the MHC class I pathway.

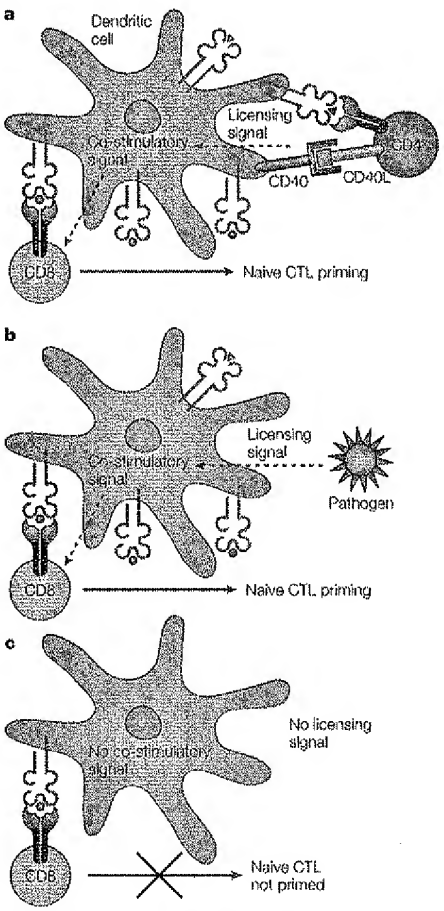

Figure 2 | **Licensing of dendritic cells is required for the generation of CTL immunity. a** | To prime naive cytotoxic T lymphocytes (CTLs), dendritic cells (DCs) first require a helper T cell-dependent signal via CD40/CD40L (CD154) (REFS 8–10). **b** | Such CD40-dependent licensing is, however, not always necessary, since pathogen-derived signals, such as viral products, can also license DCs[8]. In either case, the DCs must be licensed before they can prime naive CTLs. In the absence of licensing, naive CTLs cannot be primed by DCs (c).

The second definition referred to the capture and representation of cell-associated antigen in either the MHC class I or MHC class II pathways. Both definitions have their merits, but it is time to choose which should be used. The most common view seems to be that cross-presentation describes the processing of exogenous antigen into the MHC class I pathway. In this case, cross-priming and cross-tolerance can only really be used in reference to the response of CTL and not helper T cells, which is somewhat of a limitation, but acceptable. As discussed below, this is a property that is primarily limited to a subset of DCs[20,21]. This makes sense since the indiscriminate capacity of all cells to present exogenous antigens in the MHC class I pathway (cross-presentation) would potentially target non-infected tissue cells that endocytosed viral debris, for destruction by virus-specific CTLs. Therefore, DCs have been bestowed with a specialized property that allows them to cross-present antigens derived from other cells, for the stimulation of naive CTLs (FIG. 1c).

Identity of the cross-priming APC

Despite the discovery of cross-priming in the mid-1970s[19], the phenotype of the APC responsible for this process remained elusive for a quarter of a century. Although this cell was clearly of bone marrow origin[22,23], and a professional APC (that is, able to activate naive T cells)[18], researchers had been unable to directly identify the specific subset responsible for cross-presentation. Several groups provided evidence that DCs, macrophages and even B cells were able to cross-present antigens *in vitro* under specific circumstances[24–29], but little success was derived from *in vivo* attempts to isolate the cross-presenting APC[30]. Recently, however, Bevan and co-workers[21] examined the three known splenic DC subsets (TABLE 1) and provided the first evidence that $CD8^+$ DCs are responsible for cross-priming *in vivo*. In these studies, mice were injected with ovalbumin (OVA)-bearing cells (known to induce CTLs by cross-priming[31]), left for 14 hours to allow their DCs to capture and process antigen, and then DC subsets were isolated from the spleen and examined *in vitro* for their capacity to activate OVA-specific CTLs. Only the $CD8^+$ DC subset cross-presented OVA under these conditions, despite evidence of antigen capture by all three DC subsets. Shortman and colleagues[20] later reported that soluble OVA injected intravenously was also cross-presented by $CD8^+$ DCs. Again, all three subsets of splenic DCs captured OVA, but only the $CD8^+$ subset cross-presented it to CTLs. Interestingly, double-negative DCs ($CD8^-CD4^-$) could be induced to cross-present OVA if exposed to the bacterial product, lipopolysaccharide, although their cross-presenting capacity was poor compared with that of the $CD8^+$ DC subset.

So, why has it taken so long to identify $CD8^+$ DCs as the cross-priming APC? First, $CD8^+$ DCs were themselves only identified as a DC subset in 1992 (REF. 32), they were undetected prior to this because of their sensitivity to isolation procedures. Second, the capacity of this subset to capture antigens *in vivo* and then cross-present them *in vitro* is very inefficient[21]. Therefore, fragility combined with *in vitro* presentation inefficiency frustrated early attempts at identifying the cross-presenting APC. Although $CD8^+$ DCs now seem to be the predominant cross-presenting subset, the presence of lipopolysaccharide did allow cross-presentation by double-negative DCs[20]. So, perhaps different DC subsets will cross-present under different conditions.

As discussed in detail below, cross-presentation is associated with both immunity and tolerance. So far, identification of the tolerogenic cross-presenting APC has not been achieved, although Kurts and colleagues[33] recently reported that this cell is $CD11c^+$, supporting the idea that it is of DC origin. More precise phenotypic definition of this DC subset must await future studies.

Table 1 | **Phenotype of the three subsets of DCs in the spleen**

Phenotype				References
CD4/CD8	$CD8^+$	$CD8^-CD4^-$	$CD4^+$	80
Mac-1	–	+	+	
DEC205	+	–	–	
Function				
Phagocytic	+	+	+	81
Pinocytic	+	+	+	20
Cross-presentation of soluble OVA	+	– (+ with LPS signal)	–	20
Cross-presentation of cell-associated OVA	+	–	–	21
Location				
Location (resting)	T-cell area	Marginal zone	Marginal zone	82,83
Location (after LPS)	T-cell area	T-cell area	T-cell area	82

LPS, lipopolysaccharide; OVA, ovalbumin.

Antigens and cross-presentation

How exogenous antigens enter the MHC class I pathway has been extensively reviewed elsewhere[34], but three general mechanisms have been defined. The first involves direct 'injection' of pathogen-derived antigenic material into the cytosol of host APCs (mediated by viruses[35] and some bacteria, such as *Listeria monocytogenes*[36]), which allows processing of pathogen proteins by the normal cytosolic machinery for MHC class I. The second mechanism involves endosomal processing. This consists of either direct endosomal loading of preformed MHC class I molecules with peptide determinants that are generated in the endosomal compartment[37,38] or 'regurgitation' of peptide antigen from the endosomal compartment onto the cell surface for association with preformed MHC class I molecules[39]. The third mechanism of cross-presentation involves 'cytosolic diversion' by as yet undefined pathways. In this case, exogenous proteins are diverted from either the endosomal compartment or directly from the extracellular fluid into the cytosol for processing in the conventional MHC class I pathway. This third situation might be represented by a single mechanism or several different processes, but includes cross-presentation of heat-shock proteins[40], antibody complexes[25], exosomes[41,42], apoptotic cells[29], necrotic cells[43] and macropinocytosis[24].

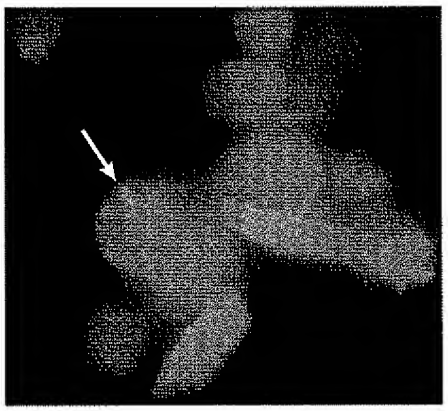

Figure 3 | **Monkey dendritic cells (DCs) acquire labelled plasma membrane from other live monkey DCs.** DCs, produced from monkey $CD14^+$ monocytes by culture in granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 for 4 days, were labelled with either 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodi-carbocyanine perchlorate (falsely coloured green) or 5-chloromethylfluorescein diacetate (falsely coloured red) and co-cultured in a five cell microscopy chamber[56]. This image was collected at 160 minutes. As single labelled cells interact, small amounts of label are transferred for donor cells to recipient DCs. The arrow indicates an example of transferred material. We thank Dr S. Barratt-Boyes and Dr L. Harshyne for providing this image.

Although there are several pathways for cross-presentation, our current understanding of which pathway(s) operate *in vivo* for cross-presentation of cell-associated antigens derived from virus-infected cells or self tissues is minimal. Many types of protein antigens have been reported to be cross-presented, including nuclear[44], cytoplasmic and cell surface[45], foreign[31] and self[15], as well as viral[46], bacterial[31] and eukaryotic[19]. The level of expression by the donor cell seems to be very important for successful cross-presentation[47]. Expression levels crucially dictate whether sufficient antigen will be cross-presented to stimulate CTLs. Under normal circumstances, cross-presentation is probably less efficient than direct presentation, since cross-presentation requires the additional step of transfer from one cell to another. So, to detect cross-presentation in a model system, it is important that the donor tissue expresses sufficient antigen (for an extended discussion of this important issue see REF. 30).

Apoptotic cells have been reported to be a good antigen source for cross-presentation *in vitro*[29,48,49], and whereas necrotic cells were initially thought to be excluded from cross-presentation[29], recent studies show that this is not the case[43]. Although there is some evidence that necrotic cells can cross-prime *in vivo*, as illustrated by the capacity of sonicated cell debris to induce CTLs[50], there is no direct *in vivo* evidence that apoptotic cells are cross-presented. McPherson and colleagues[51] report that a subset of rat DCs constitutively carry apoptotic gut epithelial cells to the mesenteric lymph

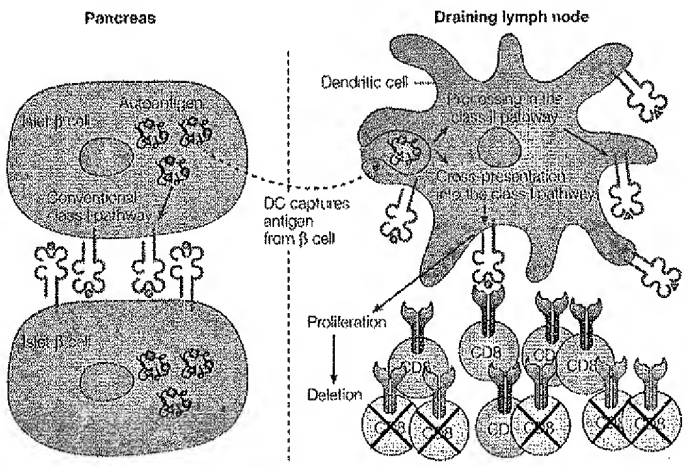

Figure 4 | **Cross-presentation of self-antigens leads to induction of CTL tolerance to peripheral tissues.** Dendritic cells (DCs) capture antigen from peripheral tissues, such as pancreatic β-cells, and then cross-present them to autoreactive cytotoxic T lymphocytes (CTLs) in the draining lymph node. This leads to proliferation followed by deletion of the autoreactive CTL, resulting in tolerance to the self-antigens. At present, it is unclear whether the DC captures antigen directly from the tissue cells or simply resides in the draining lymph node where it captures shed antigens.

nodes. They have yet to examine antigen presentation, but their studies formally show that DCs can capture and transport apoptotic cells *in vivo*. In our own studies[17], pancreatic islets that transgenically express low amounts of OVA failed to supply antigen for cross-presentation in the draining lymph node, unless they were first exposed to activated CTLs that destroyed islet tissue. Therefore, killing islet cells enhanced cross-presentation. Whether this was due to the generation of apoptotic cells or simply due to the release of antigen has yet to be resolved[52].

Despite the evidence that cellular destruction can facilitate cross-presentation, there are situations in which neither apoptosis nor necrosis seem necessary. For example, several lines of transgenic mice express antigens in the pancreatic islets that are cross-presented in the draining lymph nodes in the apparent absence of apoptosis[47,53,54]. So, how can cross-presentation occur without cellular destruction? One simple possibility is that antigens are secreted and then captured in the draining nodes by DCs that are capable of cross-presentation. However, careful analysis of the response to soluble versus cell-associated OVA has led us to conclude that even when islets express secreted OVA, cross-presentation requires that the antigen is captured in a cell-associated form, perhaps directly from the islet cells[55]. Support for a mechanism in which DCs can directly capture cellular antigens from live cells comes from a recently described process we refer to as 'nibbling'[56]. In these studies, DCs were cultured with various other cell types labelled with dyes. In all cases, material was captured by unlabelled DCs through the nibbling of small vesicles from the donor cells. An example of this transfer is shown in FIG. 3. This was found to be DC-specific, with no evidence for nibbling by non-DC cell types, such as macrophages. These observations support the possibility that DCs resident in the tissues might move from cell to cell, nibbling pieces of tissue cells without causing damage. This material could then be transported to the draining lymph node for cross-presentation to naive T cells.

CENTRAL TOLERANCE
The generation of tolerance to self during T-cell development in the thymus.

Role of cross-presentation in self-tolerance

As well as providing a mechanism for generating immunity to intracellular infections, cross-presentation has been reported to participate in tolerance induction. Von Boehmer's laboratory first reported a role for cross-presentation in CENTRAL TOLERANCE[57], showing that minor histocompatibility antigens were cross-presented within the thymus, where they tolerized CTLs; a process later shown to occur by deletion[58]. They referred to this process as 'cross-tolerance'. More recently, cross-tolerance was observed for antigens that were expressed extra-thymically in organs, such as the pancreas and kidney[3,59]. These studies were initiated by the discovery that pancreatic and renal antigens can be constitutively cross-presented in draining lymph nodes by a bone marrow-derived APC[45], most probably a DC[53]. Such cross-presentation induced proliferation of naive CD8 T cells, but ultimately led to their deletion[3] (FIG. 4). In related studies, helper T cells were shown to be tolerized by MHC class II-restricted presentation of tissue-derived antigens[59,60]. Although in this case it is not strictly cross-presentation, since antigens enter the conventional MHC class II pathway, the tolerance process is most probably mediated by the same DCs through the same tolerogenic signals.

Antigen expression levels significantly determine whether self-antigens are cross-presented in sufficient amounts to cause CTL deletion[53]. So, antigen dose strictly determines the state of tolerance to self, with high-dose antigens inducing deletional tolerance by cross-presentation, and low-dose antigens being ignored. Even when sufficient antigen is expressed to cause cross-presentation, the rate of deletion might be affected by dose. Sherman and colleagues[54] reported the more rapid deletion of haemagglutinin-specific CTL in homozygous compared with heterozygous transgenic mice, due to increased expression of haemagglutinin in the pancreas. Interestingly, they showed the deletional process to be rather slow, with deletion of 10^5 adoptively transferred CTLs requiring 2–4 weeks. This rate would, however, be more than adequate for dealing with the few self-reactive CTLs expected to be generated in a normal repertoire.

In addition to antigen dose, the site of expression and age of the host influence cross-presentation of self-antigens[61], and hence cross-tolerance[62]. This understanding began with the observation that presentation of an islet antigen in non-obese diabetic mice did not begin until about the third week of life[61]. Similarly, both OVA[61] and haemagglutinin[62], transgenically expressed in the islet β-cells, failed to be cross-presented until this time. This did not seem to be due to a lack of antigen expression[62], supporting the idea that cross-presentation

ALLOGENEIC
Individuals within a species that express allelically variant genes that lead to rejection of transplanted tissue.

of pancreatic antigens is suppressed during the juvenile period. However, this is not a general phenomenon for all tissues since OVA expressed in the kidney was cross-presented at the earliest time examined (day 10) (REF. 61). An important consequence of the late onset of cross-presentation of pancreatic antigens is that there is no deletion of islet-specific CTLs in young mice. In mice expressing haemagglutinin as a transgenic antigen in the pancreatic islets, for example, infection of juvenile mice with influenza virus induced haemagglutinin-specific CTLs that caused autoimmune diabetes[62]. This was not the case for adult mice, which had deleted their haemagglutinin-specific CTLs by cross-presentation and were resistant to diabetes induction by influenza virus infection. These observations raise the important question of whether juvenile diabetes in humans is associated with a similar failure to cross-present islet antigens during our early years of life. These data indicate that cross-presentation of tissue antigens, and hence cross-tolerance, can be regulated both temporally and regionally. How this regulation occurs must now be addressed.

Overall, several studies clearly show that cross-presentation can lead to peripheral self-tolerance[3,54,59,60,62]. The important unanswered questions are: (i) how are T cells tolerized, (ii) what is the DC subset responsible for this tolerance induction and, most importantly, (iii) does this form of tolerance have a significant role in the natural maintenance of self-tolerance? With respect to the last question, most studies have used transgenically expressed antigens to monitor peripheral tolerance, and it will be important to verify the role of cross-presentation in self-tolerance to natural antigens.

Role of cross-presentation in viral immunity

Cross-presentation was first discovered because of its role in generating CTL immunity (cross-priming) to minor histocompatibility antigens expressed by transplanted ALLOGENEIC cells[19]. Other cell-associated antigens, including those expressed by tumours[21] and viruses[46], have been reported to cross-prime. However, there are few studies that unequivocally show cross-priming to be vital for natural, protective, CTL immunity. This does not mean that cross-priming has no role in natural immunity, only that it remains difficult to discriminate between the role of cross-presentation and direct presentation in natural CTL priming. In this review, we have focused on the role of cross-priming in viral immunity, although there is good evidence that this mechanism also participates in tumour immunosurveillance (BOX 1).

Over the years, it has been shown that many viral antigens can be cross-presented[22,53,63,64,66–68], although in these cases viral proteins were not expressed during virus infections but by transformed or transfected cells, or within transgenic mice. Virus-specific CTL immunity has been shown to depend on bone marrow-derived cells (presumably DCs) for several infections, including influenza virus[65], vaccinia virus[46,66], poliovirus[46] and lymphocytic choriomeningitis virus[65], consistent with a role for cross-priming in viral immunity. In these cases, however, it is difficult to exclude direct presentation by virus-infected DCs.

Irrespective of their preferred cell tropism for productive replication, many viruses have a broader range of infectivity, in which even partial or abortive infections can give rise to perfectly good CTL determinants. Consequently, active measures are required to unambiguously exclude the possibility of direct presentation, even when a virus is not formally known to infect DCs. In one attempt to exclude direct presentation, Norbury and colleagues[67] used a combination of chemical and UV treatment to confine infection to an introduced non-haemopoietic cell line, and thus demonstrate virus-specific CTL cross-priming. Probably the most convincing evidence that cross-priming is sufficient to prime CTL immunity during virus infection, however, comes from Rock and colleagues[46]. They provided direct evidence for CTL cross-priming to poliovirus in a murine model, in which direct infection of DCs was made impossible. Mice are not a natural host of poliovirus as they lack the receptor found on human cells necessary for virus infection. But by transgenically expressing this receptor on only the non-bone marrow-derived compartment, Sigal and co-workers[46] were able to ensure that bone marrow-derived APCs could not be infected. The demonstration that CTL immunity required antigen presentation by a bone marrow-derived cell, despite the inability of poliovirus to infect such cells, implicated cross-priming in the induction of CTL immunity to this virus. Although this example directly shows that virus immunity can be mediated by cross-priming, it does not address the contribution of cross-priming versus direct priming when both pathways are available. Therefore, it remains a formal possibility that cross-priming was observed only because other pathways were inaccessible.

Overall, these studies illustrate that cross-priming is observed extensively in experimental systems, supporting the idea that this process exists and is likely to be important for natural infections. One can envisage

Box 1 | Cross-priming and immunity to tumours

For generating cytotoxic T lymphocyte (CTL) immunity to tumours there are really only two possibilities; either the CTL are primed by direct recognition of antigen on the tumour cells, or else the tumour antigens are cross-presented on host dendritic cells. At present, there is a growing body of evidence that indicates cross-priming can induce immunity to tumours[3,21–23,53,63,64,66–68], and an equally convincing set of data showing it cannot[69–71]. In some cases, in which cross-priming is absent, CTL immunity might be generated by allowing access of the tumour to the secondary lymphoid compartment for direct encounter with naive T cells[72–74]. Whether cross-priming is involved in the natural immunosurveillance of spontaneous tumours remains unresolved. Certainly, some tumours are induced by viruses, raising the possibility that, at least in these cases, virus-specific cross-priming could limit tumorigenesis. However, it is difficult to assess the significance of cross-priming in natural tumour immunity, since detectable tumours must have escaped immunosurveillance to arise. So, although failure of some tumours to cross-prime could be taken as evidence that cross-priming is not involved in natural tumour immunity, it could equally well mean that tumours only arise if they subvert cross-priming. At present, we cannot distinguish between these two alternatives.

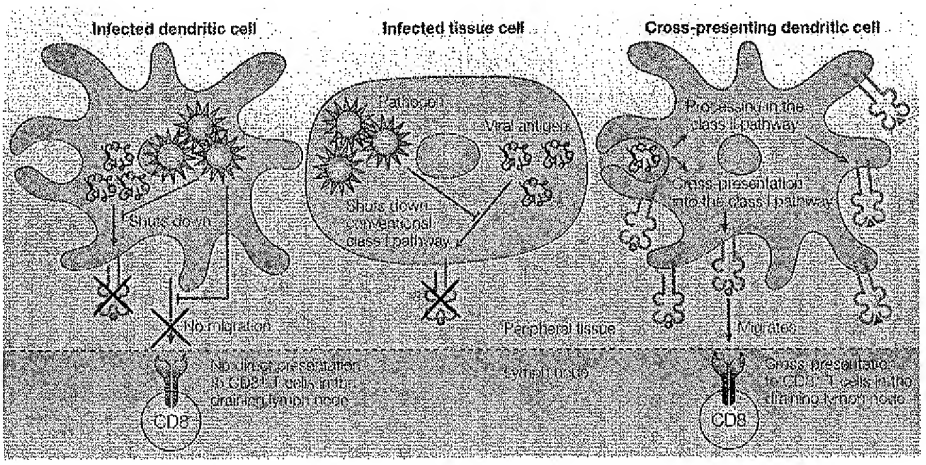

Figure 5 | **Viral subversion of dendritic cell function; cross-priming is required to generate CTL immunity.** Some viruses impair dendritic cell (DC) function during infection, for example by inhibition of MHC class I-restricted presentation or blocking migration of DCs. In these circumstances, viral inhibition of infected DCs prevents cytotoxic T lymphocyte (CTL) stimulation by the direct MHC class I pathway. However, cross-presentation of antigens that are derived from infected cells by uninfected DCs is likely to result in CTL immunity in the face of inhibitory mechanisms. Although virus-mediated inhibition of target-cell expression of MHC class I will also impair the effector phase of responses induced by cross-priming, this is unlikely to completely block recognition by effector CTLs.

that cross-priming would have an important role in cases in which a virus infection is truly localized to a peripheral non-lymphoid compartment, such as for papilloma virus, in which the infection is confined to the epithelial cells of the skin[68]. In addition, cross-priming could be important in instances where viruses have evolved mechanisms that specifically inhibit conventional MHC class I-restricted antigen processing and presentation[69–75]. In these situations, cross-presentation would result in successful CTL priming in the face of inhibitory mechanisms that would otherwise prevent direct presentation by infected DCs. In addition to the specific targeting of antigen presentation, there are emerging examples of viruses that inhibit various aspects of DC function. Recently, several viruses, including herpes simplex virus[11], measles virus[12,13], retrovirus[14], canarypox virus[15], vaccinia virus[16] and lymphocytic choriomeningitis virus[17], have all been shown to have detrimental effects on DCs. Given that professional APCs, such as DCs, are essential for priming viral responses, but that some of these same viruses (for example, vaccinia virus) can 'deactivate' DCs during infection, the logical conclusion is that immunity is induced by cross-priming (FIG. 5). By cross-presenting viral antigens, there is no requirement for DCs to be infected (and exposed to the associated hazards) in order to prime naive CTLs to MHC class I-restricted viral antigens. It therefore seems that two different reasons could justify the maintenance of cross-priming by the immune system: first, as suggested by Bevan[18], it might be very important for generating CTL immunity to tissue-specific viruses that do not infect DCs; and additionally, it might be vital for generating immunity to viruses that infect DCs, but then inhibit their function.

The current verdict on cross-presentation

Over the past decade, a number of significant steps have been taken towards understanding cross-presentation and its consequences — cross-priming and cross-tolerance. Building on the earlier efforts of Bevan and many others, Pardoll and co-workers clearly showed a role for cross-priming in immunity to experimental tumours, whereas Rock's lab generated the first convincing evidence for virus-specific cross-priming. Extending the studies of von Boehmer's lab on thymic cross-tolerance, several groups provided strong evidence that peripheral tissue antigens could be cross-presented in the draining lymph nodes for induction of peripheral self-tolerance. In addition, important observations have been made about the nature of antigenic material targeted for cross-presentation, including evidence that cross-presentation can be targeted by heat-shock proteins, apoptotic and necrotic cells, exosomes or even immune complexes. Finally, with the advent of methods for the purification of DC subsets, largely pioneered by Shortman and colleagues, Bevan's lab was again able to make a significant impact in the field of cross-priming 25 years after his original discovery, by reporting that it is primarily the $CD8^+$ DC subset that is responsible for this process. Despite all this new and exciting information, our understanding of cross-presentation is still only in its infancy. Our new knowledge of which DC subsets are responsible for this process will help us enormously in future efforts to gain a detailed understanding of the mechanism of cross-presentation. Finally, it will be the design of experimental models that can address the nature of tolerance to natural self-antigens, and the contribution of direct versus cross-presentation to immunity, that will be important if we are to define the extent to which we depend on cross-presentation for immunity and tolerance.

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Online links

DATABASE LINKS
The following terms in this article are linked online to:
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
CD4 | CD8 | CD40 | CD40L | CLIP | GM-CSF | Herpes simplex virus | Measles virus |TAP | Vaccinia virus
Access to this interactive links box is free online.

Biogs

William R. Heath received his Ph.D. from the University of Melboune in 1988 and then spent several years at Scripps Research Foundation in La Jolla before moving to the Walter and Eliza Hall Institute in 1990. Since then, his studies, in collaboration with Dr Carbone, have focused on understanding the mechanisms of peripheral tolerance, particularly with respect to cytotoxic T lymphocytes (CTL). This led to the observation that tissue antigens are cross-presented and induce deletional tolerance. He has also been responsible for dissecting the requirements for cross-priming, providing the first evidence that CD40 licensing of dendritic cells is important for their capacity to prime CTL.

Francis R. Carbone received his Ph.D. from the University of Melboune in 1985. He spent a postdoctoral period in the US at Scripps Research Foundation where, in Dr Michael Bevan's lab, he described the cytosolic class I processing pathway and the capacity of exogenous protein antigens to cross-prime *in vivo*. He returned to Australia in 1990 and is currently an Associate Professor at the Department of Microbiology and Immunology, University of Melboune. Dr Carbone has a long-standing interest in cross-presentation and is currently applying this to the study of herpes simplex virus infection.

At a glance summary

T lymphocytes can be separated into two subpopulations, based on their expression of CD4 and CD8. The $CD4^+$ subset is primarily responsible for providing help to other immune cells, whereas $CD8^+$ T cells are best known for their capacity to kill virus-infected cells.

Cross-presentation is defined as the processing of exogenous antigens into the major histocompatibility complex (MHC) class I pathway. Cross-priming and cross-tolerance refer to the induction of cytotoxic T lymphocyte (CTL) immunity or tolerance, respectively, that is induced by cross-presented antigens.

Despite the discovery of cross-priming in the mid-1970s, the antigen-presenting cell responsible for this process has only recently been identified. Bevan and co-workers provided evidence that it is the $CD8^+$ dendritic cell (DC).

Although there are several pathways for cross-presentation, our current understanding of which pathway(s) operate *in vivo* for cross-presentation of cell-associated antigens that are derived from virus-infected cells or self tissues is minimal.

As well as providing a mechanism for generating immunity to intracellular infections, cross-presentation has been reported to participate in tolerance induction. Such cross-tolerance is most probably mediated by DCs and leads to the deletion of self-reactive CTLs.

Antigen expression levels, the site of expression, the time of expression and the availability of help, crucially determine whether self-antigens cause cross-tolerance.

There are few studies that unequivocally show cross-priming to be crucial for natural, protective, CTL immunity. This does not mean that cross-priming has no role in natural immunity, only that it remains difficult to discriminate between the role of cross-presentation and direct presentation in natural CTL priming.

Virus-specific CTL immunity has been shown to depend on bone marrow-derived cells (presumably DCs) for several infections, including influenza virus, vaccinia virus, poliovirus and lymphocytic choriomeningitis virus, consistent with a role for cross-priming in viral immunity.

One can envisage that cross-priming has an important role in cases where a virus infection is localized to a peripheral non-lymphoid compartment, such as for papilloma virus infection of the epithelial cells of the skin. In addition, cross-priming could be important where viruses have evolved mechanisms that specifically disrupt the immune functions of DCs.

Links

CD4
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=920
CD8
http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi?Q=cd8%20not%20cd1a%20not%20il10%20not%20Igsf6&ORG=Hs
CD40
http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=958
CD40L
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